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14. ABSTRACT

Our studies show that the ligand binding domain (LBD) of estrogen receptor alpha (ERα) is important in mediating ICI 182,780 (ICI)-induced degradation of ERα. Further, we show that a single residue within helix 12 (H12) is specifically important in allowing for the degradation of ERα following treatment with ICI. That this residue is not conserved between ERα and ERβ may explain why ERβ does not undergo rapid ICI-mediated degradation, and may also explain why ICI is not as effective at reducing the basal activity of ERβ. This study is a key first step in elucidating the mechanism by which ERα undergoes ligand-mediated degradation, and hopefully future studies will enable the identification of points in the degradation pathway that can be exploited pharmacologically for the treatment of breast cancer, and potentially other malignancies that are dependent upon estrogen signaling for continued growth.

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Estrogen receptor alpha, protein stability

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Introduction

Estrogens make a major contribution to the growth and progression of estrogen receptor (ER) positive breast cancer. The actions of estrogen (E2) are mediated primarily through binding to either of the two isoforms of ER, ERα and ERβ [1, 2]. This binding induces a conformational change in the receptor that leads to receptor dimerization, DNA binding, recruitment of coregulatory proteins, and the modulation of target gene transcription. The specific E2-induced conformational change of ERα also leads to receptor degradation through the 26S proteosome pathway, an event that is thought to be intimately linked to transcriptional activity of this receptor [3, 4]. In contrast, binding of E2 to ERβ does not lead to rapid degradation, despite inducing efficient transcriptional activation. This suggests that there are conformational differences between E2-bound ERα and E2-bound ERβ and they are responsible for dictating the recruitment of proteins involved in receptor degradation. Interestingly, the pure anti-estrogen ICI 182,780 (ICI) also elicits degradation of ERα, but not ERβ, again supporting the notion that the ligand-mediated structural changes in ERα and ERβ are distinct and thus lead to different biological outcomes [5]. Although both E2- and ICI-mediated degradation of ERα require the 26S proteosome pathway, different proteins are required for the two processes, making it clear that the mechanisms are distinct [6]. Studies have implicated residues within helix 12 (H12) of the ligand binding domain (LBD) of ER as important in determining ligand-mediated stability [7-9]. Thus, our goals for this project were to (1) define the role of the LBD of ERα in dictating receptor stability, (2) identify proteins involved in ligand-mediated degradation by screening for proteins that interact with the residues of H12 or with E2- or ICI-bound ERα, and (3) determine the functionality of these identified proteins.

From a clinical standpoint, the results of this study will lend insight into the mechanism of action of ICI, which is currently in clinical use. By uncovering the exact mechanism by which ICI treatment leads to ERα degradation, we can exploit this pathway pharmacologically to enhance the removal of ERα from breast cancer cells. Further, since ICI exhibits poor pharmacokinetic (PK) properties and many breast tumors develop resistance to ICI therapy, mapping the pathway from ICI treatment to ERα protein turnover will enable the development of novel pharmaceuticals with improved PK profiles and the ability to reduce ERα levels in breast tumor cells, and more importantly are not cross-resistant with ICI.

Body

The first task for this project was to evaluate which regions of estrogen receptor alpha (ERα) were necessary for ligand-mediated degradation. Preliminary studies by our lab and others suggest that the ligand binding domain (LBD), and specifically helix 12 (H12) within the LBD, is a crucial region in determining the stability of ERα, therefore we began our studies by investigating the role of the LBD in mediating ligand-induced degradation. There were a few considerations to be addressed before beginning these studies, namely to identify a model system. It was important to use a cell line for our studies that was known to contain the machinery necessary for ERα degradation, and considering our interest in breast cancer we were interested in using a breast cancer cell line. Therefore, we chose to use the ERα-positive MCF7 breast cancer cell line for our studies. As shown in **Figure 1A**, ERα undergoes rapid degradation upon treatment with either the agonist 17β-estradiol (E2) or the pure antagonist ICI 182,780 (ICI). In setting up our model system, we also needed a way to study the requirements for each region of ERα for turnover. To accomplish this, we exploited the fact that ERβ does not undergo rapid ligand-mediated degradation. We reasoned that any region necessary for degradation of ERα would be transferable to ERβ and the resulting chimera would undergo ligand-mediated degradation in a pattern similar to wild-type ERα. However, MCF7 cells do not express ERβ; therefore an N-terminal V5 tagged version of ERβ was cloned for use in these studies. As shown in **Figure 1B**, V5-ERβ does not undergo ligand-mediated degradation after 4 hours of E2 or ICI treatment.

The first ERα/ERβ chimera tested contained the A/B, C, D, and F domains of ERβ with the LBD of ERα and was thus denoted ERβ-αLBD (see **Figure 2A**). We first evaluated the transcriptional activity of this chimera by transiently expressing it in the ER-negative HeLa cell line along with an ERE-luciferase reporter. We show that ERβ-αLBDs is transcriptionally active and behaves similarly to both ERα and ERβ in response to E2 treatment. Interestingly, while the transcriptional activity of ERβ is not suppressed below basal by ICI, the activity of ERβ-αLBD was decreased upon treatment with ICI (**Figure 2B**), suggesting this characteristic of ERα had indeed been transferred to ERβ within the ERα LBD. This also suggested that residues important for the suppression of basal receptor activity by ICI are contained within the LBD of ERα, and that these residues are not identical in the ERβ LBD. We then investigated the stability of the ERβ-αLBD chimera in MCF7 cells and found that this chimera undergoes degradation upon treatment with ICI, but not with E2 (**Figure 2C**). From these data we concluded that the LBD of ERα is able to induce degradation upon treatment with ICI.

Next we sought to determine which residues within the LBD are important in mediating ICI-induced ERα degradation. Given the documented importance of H12 in regulating receptor stability, we aligned the sequences of ERα and ERβ in the H11/H12 region (**Figure 3A**). There are only two residues that differ between the two receptors in this region, at position 487 within the H11/H12 linker and position 496 within H12 of ERβ. Therefore we employed site-directed mutagenesis on ERβ to change these residues to the corresponding amino acid in ERα (valine 487 to leucine (ERβ V487L) and asparagine 496 to aspartic acid (ERβ N496D)) and examined the transcriptional activity and protein stability of these mutated forms of ERβ. Interestingly, we found that mutation of either site in ERβ to the corresponding residue of ERα resulted in suppression of basal transcriptional activity in the presence of ICI (**Figure 3B**) as compared to wild-type ERβ. Further, ERβ N496D was rapidly degraded in the presence of ICI (**Figure 3C**). This change was independent of any effect on the transcriptional activity or protein stability of ERβ in the presence of E2. Preliminary studies suggest that ERβ V487L does not exhibit altered protein stability in the presence of E2 or ICI as compared to wild-type ERβ, although further work is necessary before any conclusion can be definitively drawn.

Taken together, our data suggest that the LBD of ERα is responsible for ICI-mediated degradation of ERα, and further that D545 within H12 is sufficient to induce ER degradation in the presence of ICI. Binding of ICI to ER elicits a conformational change that dictates receptor stability by modulating protein-protein interactions and surface hydrophobicity. Differences in the LBD of ERα and ERβ allow for distinct conformational changes to occur upon ICI binding, thus leading to different patterns of receptor degradation. Although the global conformation of the ER LBD may play a role in determining the stability of ERα and ERβ in the presence of different ligands, our studies suggest that in the case of ICI, the differences in stability can at least partially be pinpointed to a change in polarity at one particular residue within H12.

The identification of a single residue within H12 of ERα that plays a critical role in allowing for ICI-mediated degradation will allow us to embark on the second specific aim of this study, namely to identify proteins that interact with this region of ERα and thus may play a role in mediating receptor turnover. To accomplish this, we will compare the protein binding profiles of wild-type ERα with a mutant form of ERα in which D545 has been changed to the corresponding residue in ERβ.

In the course of studying the degradation of ERα by various ligands, we and the Manglesdorf laboratory at UT Southwestern have characterized a novel endogenous ER ligand, 27-hydroxycholesterol (27HC), that also induces receptor degradation. This finding of an endogenous ligand outside the three estrogens that regulates ER function is in itself of great significance. At this point studies are underway to further characterize 27HC as an ER ligand. We intend to determine if the mechanism of 27HC-bound ERα degradation is similar to that of E2-bound or ICI-bound ERα degradation. It is our hypothesis that 27HC, which is produced at high levels by macrophages, impinges on estrogen receptor signaling in the breast and may affect the development or progression of

breast cancer, especially in post-menopausal women. Studies show a higher risk of breast cancer in this subgroup of patients, as well as obese/hypercholesteremic patients, where there is a significant product-precursor relationship between cholesterol and 27HC. Together this makes establishing the role of this novel endogenous ligand important with implications that it may functionally interact with other ER ligands, such as Tamoxifen, that are currently used for breast cancer treatment, or 27HC itself may be a novel pharmacological target for treatment of this disease.

Key Research Accomplishments
- Transiently expressed ERβ does not undergo ligand-induced degradation in MCF7 breast cancer cells
- The LBD is important in mediating the degradation of ICI-bound ERα
- D545 within H12 of the LBD is important in ICI-bound ERα degradation

Reportable Outcomes
Molecular determinants of estrogen receptor alpha stability. DuSell CD, Wittmann BM, Chang CY, and DP McDonnell. Poster presented at Keystone Symposia Nuclear Receptors: Steroid Sisters, Banff, Alberta (March 18 – 23, 2006).

DuSell CD, Umetani M, Shaul PW, Mangelsdorf DJ, and DP McDonnell. 27-Hydroxycholesterol in an endogenous Selective Estrogen Receptor Modulator (SERM). Manuscript in preparation.

Conclusion
Our studies show that the LBD of ERα is important in mediating ICI-induced degradation of ERα. Further, we show that D545 within H12 is specifically important in allowing for the degradation of ERα following treatment with ICI. That this residue is not conserved between ERα and ERβ may explain why ERβ does not undergo rapid ICI-mediated degradation, and may also explain why ICI is not as effective at reducing the basal activity of ERβ. This study is a key first step in elucidating the mechanism by which ERα undergoes ligand-mediated degradation, and future studies will enable the identification of points in the degradation pathway that can be exploited pharmacologically for the treatment of breast cancer and potentially other malignancies that are dependent upon estrogen signaling for continued growth.

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Appendices
None.

Supporting Data

A ERα

V   E2   ICI

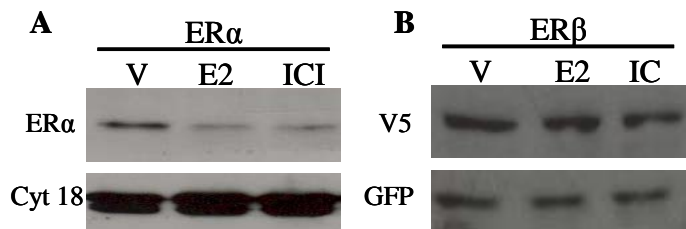ERα

Cyt 18

B ERβ

V   E2   IC

V5

GFP

Figure 1. ER degradation in breast cancer cells

MCF7 cells growing in phenol-red free media containing 8% charcoal stripped serum (WM) were transfected with pCDNA3.1nV5-ERβ and GFP for 24 hours prior to treatment with vehicle, E2, or ICI for 4 hours. Whole cell extract was harvested, resolved by SDS-PAGE, and subjected to immunoblotting for ERα, V5-ERβ, Cytokeratin 18 (Cyt18), or GFP.
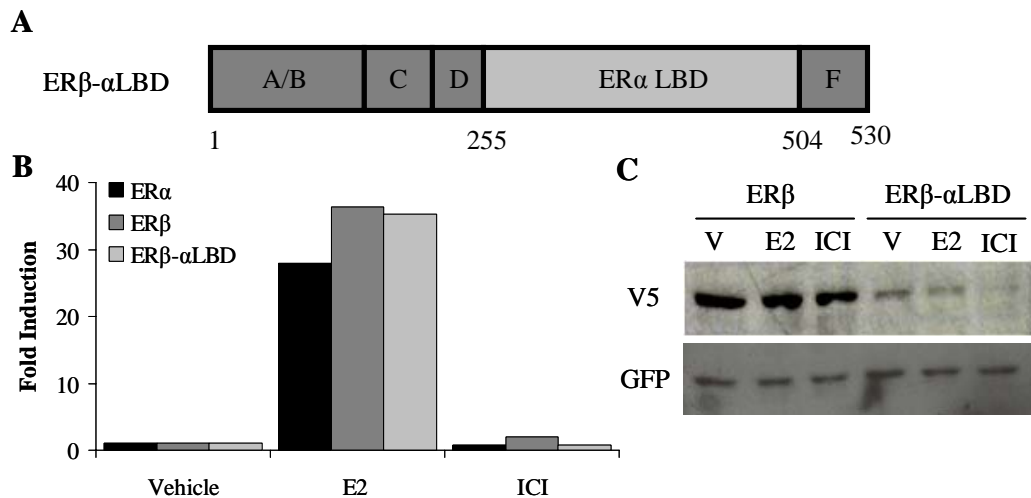
A

ERβ-αLBD | A/B | C | D | ERα LBD | F |

1   255   504 530

B

■ ERα
▨ ERβ
▨ ERβ-αLBD

Fold Induction

40
30
20
10
0

Vehicle   E2   ICI

C

ERβ   ERβ-αLBD

V   E2   ICI   V   E2   ICI

V5

GFP

Figure 2. The LBD of ERα confers ICI-mediated degradation onto ERβ

A, The ligand binding domain of ERβ was replaced with that of ERα (αLBD) to create the novel chimera ERβ-αLBD. B, pCDNA3.1nV5-ERα, -ERβ, or ERβ-αLBD were transfected into HeLa cells growing in WM along with 3XERE-TATA-luc and the CMV-β-galactosidase transfection control. After 24 hours of transfection, the cells were treated for 24 hours with vehicle, E2, or ICI, and then harvested and assayed for luciferase and b-gal. Results are presented as fold induction over vehicle. C, MCF7 cells growing in WM were transfected for 24 hours with pCDNA3.1nV5-ER or -ERβ-αLBD and GFP, treated for 4 hours with vehicle, E2, or ICI, harvested, resolved by SDS-PAGE, and subjected to immunoblotting for V5 or GFP as a transfection control.
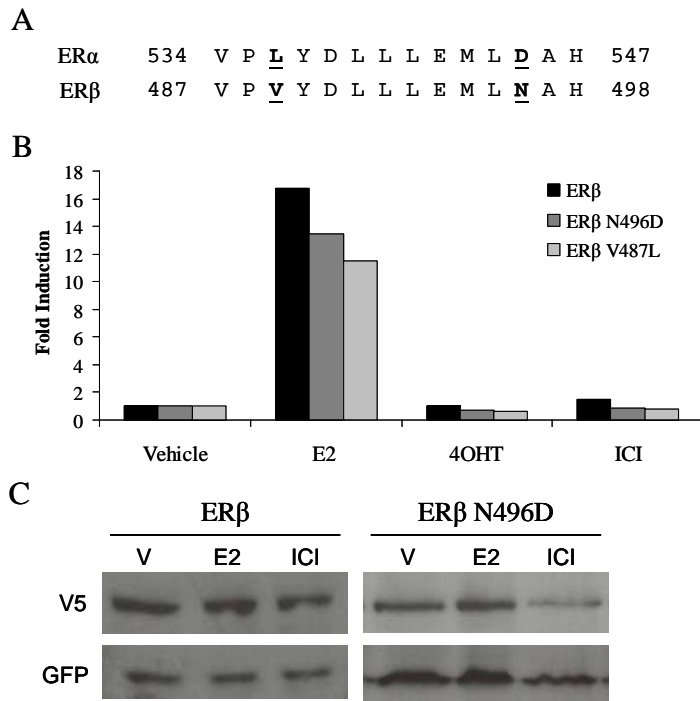
A

```
ERα    534   V P L Y D L L L E M L D A H   547
ERβ    487   V P V Y D L L L E M L N A H   498
```

B

C

Figure 3. ERβ N496D undergoes ICI-mediated degradation

A, The sequence of the H11/H12 linker region through H12 of ERα and ERβ. B, HeLa cells growing in WM were transfected for 24 hours with pCDNA3.1nV5-ERβ, -ERβ N496D, or ERβ V487L, 3XERE-TATA-Luc, and CMV-βgal transfection control. Cells were then treated with vehicle, E2, 4OHT, or ICI for 24 hours, then harvested and assayed for luciferase and bgal. Data is presented as fold induction over vehicle. C, MCF7 cells growing in WM were transfected with pCDNA3.1nV5-ERβ or -ERβ N496D and GFP for 24 hours, then treated for 4 hours with vehicle, E2, or ICI. Cells were then harvested, resolved by SDS-PAGE, and subjected to immunoblotting for V5 or GFP.